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Flow Injection Analysis System for Screening Organophosphorus Pesticides by their Inhibitory Effect on the Enzyme Acetylcholinesterase

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A flow-injection spectrophotometric procedure was developed for screening organophosphorus pesticides. The method is based on the inhibition of acetylcholinesterase immobilized on controlled porous glass beads with acetylcholine chloride as the substrate. Methyl parathion, chlorpyrifos, malathion and dichlorvos have been tested. The analytical peak height for a given acetylcholine chloride concentration correlates linearly with the logarithmic concentration of the pesticides between 1.0×10^{-3} mol L⁻¹ and 1.0×10^{-6} mol L⁻¹. If bromine water is added to the pesticide solution, a dramatic increase is observed in the analytical signal, and correlations with the logarithm of the concentrations are observed from 1.0×10^{-3} to 1.0×10^{-10} mol L⁻¹.

Keywords: pesticides, organophosphorus, acetylcholinesterase, biosensor

Introduction

Pesticides (herbicides, fungicides, insecticides) are natural or manufactured chemical compounds used in modern agriculture to exterminate or control pests; these chemicals can damage crops and livestock and reduce farm productivity. Among the various pesticides, organophosphorus insecticides (OPs) are most often applied because they exhibit low environmental persistence and display high acute toxicity to their targets.^{1,2} Organophosphates comprise a group of chemical compounds that are used extensively in agriculture as insecticides. However, overuse of OPs results in pesticide residues in food, water and the environment, which poses a significant threat to human health. These compounds express acute lethality not only to insects but also to mammals because they are potent inhibitors of acetylcholinesterase (AChE), which is an enzyme that is vital to nerve function. AChE inhibition results in the buildup of the neurotransmitter acetylcholine, which interferes with muscular responses and, in vital organs, produces serious symptoms and

eventually death. Currently, there is a universal consensus that continuous monitoring of chemical contaminants in food, water and air is required and is important to the human health.³⁻⁵

Organophosphorus pesticides can be identified and quantified by classical analytical techniques, such as gas chromatography (GC), high performance liquid chromatography (HPLC) or HPLC coupled with mass spectrometry (MS). However, these methods require time-consuming sample preparation with application of different extraction and cleanup procedures using toxic and expensive organic solvents.⁶ In laboratories where a large number of samples must be processed rapidly, a method with sufficient sensitivity that can be used for preliminary screening is a good alternative.

Over the past decade, AChE inhibition-based biosensors have emerged as simple, rapid and sensitive tools for pesticide analysis in environmental monitoring, food safety and clinical analysis.⁷⁻⁹ Therefore, several methods using biosensors based on cholinesterase inhibition for the determination of organophosphorus pesticides have been developed including potentiometric,^{10,11} conductometric,^{12,13} amperometric¹⁴⁻²⁰ and photometric²¹⁻²⁴ detectors.

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To further reduce costs, the current study focuses on the development of a simple, rapid, reliable, sensitive and low cost method for the detection of low concentrations of acetylcholinesterase inhibitors that may be present in agricultural products and natural waters.

Experimental

Method

The method is based on the determination of the acetate formed by the enzymatic reaction of AChE immobilized on glass beads with the substrate acetylcholine at a fixed concentration. The enzymatic process is depicted in Scheme 1. The acetic acid that is formed after the reaction of the acetate with sulfuric acid permeates through a polytetrafluoroethylene (PTFE) membrane and is received by an aqueous solution of bromocresol purple (BCP; 5.0×10^{-5} mol L⁻¹) causing a color change from purple ($\lambda_{\text{max}} = 590$ nm) to yellow ($\lambda_{\text{max}} = 400$ nm). The variation in the absorbance of the solution is detected spectrophotometrically at 590 nm. 1,1'-trimethylene-bis(4-formylpyridinium bromide) dioxime (TMB-4) was used to regenerate the enzyme.²²

Reagents and solutions

Acetylcholinesterase (E.C. 3.1.1.7 type VI-S from electric eel, 1000 U mg L⁻¹), methyl parathion and 1,1'-trimethylene bis (4-formylpyridinium bromide) dioxime (TMB-4) were obtained from Sigma-Aldrich (St. Louis, USA). Acetylcholine and TMB-4 solutions were prepared daily in a 0.1 mol L⁻¹ phosphate buffer (pH 7.0). Acetylcholine chloride, 99% (Acros Organics, Geel, Belgium); 3-aminopropyl-triethoxysilane, 99% (Acros Organics, Geel, Belgium); glutaraldehyde, 25%, aqueous solution (Nuclear, São Paulo, Brazil); methyl parathion, 99.5% (Supelco, Bellefonte, USA); malathion, 98% (Chem Service, West Chester, USA); chlorpyrifos, 98% (Chem Service); dichlorvos, 99.5% (Chem Service); acetone, analytical grade (Synth, São Paulo, Brazil); absolute ethanol, analytical grade, Synth; xylene, analytical grade (Carlo Erba, Italy); concentrated sulfuric acid, analytical grade (Synth); concentrated nitric acid analytical grade (Synth); sodium phosphate dibasic,

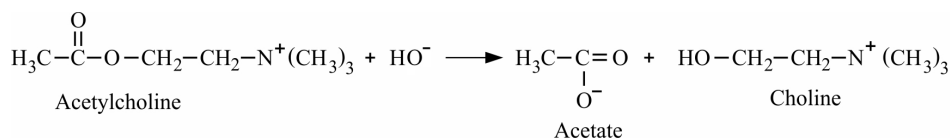
Na₂HPO₄ (Nuclear, São Paulo, Brazil); potassium phosphate monobasic, KH₂PO₄ (Nuclear, São Paulo, Brazil); soda lime with indicator (pellets) (Vetec, Rio de Janeiro, Brazil); sodium hydroxide, analytical reagent (Vetec, Rio de Janeiro, Brazil), bromocresol purple (Vetec, Rio de Janeiro, Brazil); bromine, ACS grade (Sigma-Aldrich); NaClO, analytical grade water solution, available chlorine 4-5%, from Vetec (Rio de Janeiro, Brazil); iodine, analytical grade (Synth, São Paulo, Brazil); KI, analytical grade (Synth, São Paulo, Brazil); controlled porosity glass (CPG), 240 μm, 80-120 mesh, porosity 22.6 nm beads from Sigma-Aldrich.

BCP solutions (5.0×10^{-5} mol L⁻¹) were prepared by dissolving 0.27 g of BCP in 10 mL of ethanol. Further dilutions with water were performed to obtain the desired concentration. The pH was adjusted to 7.0 by dropwise addition of a dilute 0.1 mol L⁻¹ NaOH solution. To avoid absorption of CO₂ from the air, the BCP solution was maintained in a bottle protected by a tube containing soda lime with an indicator.

The water used to prepare the solutions was first distilled in a glass distillatory, deionized in a Milli-Q Plus system and degassed prior to use.

Stock solutions of the pesticides (0.1 mol L⁻¹) were prepared in acetone by dissolving adequate quantities in 5.0 mL of acetone in a volumetric flask. When necessary, an ultrasound bath was used to promote dissolution. More dilute solutions were obtained by successive dilutions with distilled water. For example, for methyl parathion, 0.1316 g was dissolved with acetone in a 5.0-mL volumetric flask, and the volume was completed with the same solvent (concentration: 0.1 mol L⁻¹). From this solution, 500 μL was transferred to another 5.0-mL volumetric flask, and the volume was completed with distilled water solvent (concentration: 0.01 mol L⁻¹). More dilute solutions were obtained by successive dilutions with distilled water using 500 μL of a solution and completing to 5.0 mL. The same procedure was used to obtain the solutions of the other pesticides. The initial quantities were as follows: dichlorvos, 0.1105 g; malathion, 0.1652 g and chlorpyrifos, 0.1753 g. For orange juice, distilled water was substituted by the juice in the final solution that was introduced in the flow system.

The silanization of the surface of the glass beads and the immobilization of the enzyme on the CPG were performed as previously described.²²



Scheme 1.

Solutions

Acetylcholine ($5 \times 10^{-3} \text{ mol L}^{-1}$) in an aqueous buffered solution was prepared by dissolving 0.0454 g in a 50.0-mL volumetric flask and filling to the mark with Sørensen pH 7.0 buffer (0.1 mol L^{-1}). This solution was always freshly prepared prior to use.

A $5 \times 10^{-5} \text{ mol L}^{-1}$ solution of TMB-4 in an aqueous buffer was prepared by dissolving 0.0223 g in a 10.0-mL volumetric flask and filling to the mark with Sørensen pH 7.0 buffer. This solution was always freshly prepared prior use.

Saturated bromine water was prepared from elemental bromine, and the final concentration of 20 mmol L^{-1} was obtained by dilution with water. A 20 mmol L^{-1} hypochlorite solution was prepared by adequate dilution of a 4-5% solution in water. 20 mmol L^{-1} iodine was prepared by dissolving 1.27 g of solid I_2 in 250 mL of water in the presence of dissolved potassium iodide (4.0 g).

Apparatus

Peristaltic pump: Ismatec MCP; spectrophotometer: Single-beam Femto model 600; chart recorder: Cole Parmer Series 8375; flow cuvette: Hellma 1.00 cm path length quartz cuvette; sampling valve: this sampling valve has been described previously in detail;²⁵ gas diffusion cell: similar to the one previously described;^{26,27} pumping tubes: Ismatec SC0020 two-stop tubes, green-green, Tygon®, internal diameter 1.85 mm; conducting tubes: polyethylene tubes, internal diameter 1.0 mm.

Flow-injection system

The scheme of the flow injection system is shown in Figure 1. Tygon® tubing is used at the pump to push or pull solutions, and the remaining tubes of the circuit are composed of polyethylene. The solution of acetylcholine chloride (Ac) is introduced by valve (V) into the carrier stream (F_1) consisting of $5.0 \times 10^{-3} \text{ mol L}^{-1}$ Sørensen buffer (pH 7.0), which is pumped by the peristaltic pump (P) at a rate of 1.0 mL min^{-1} (rates: $F_1 = F_2 = F_3$). Then, the solution passes through the enzymatic reactor (ER) in a polyethylene tube (3.5 cm long and 3.0 mm in internal diameter) containing the immobilized enzyme. In sequence, the solution is mixed after the ER with a 0.5 mol L^{-1} sulfuric acid solution (F_2). The acetate formed in ER is mixed with the sulfuric acid solution in order to form acetic acid, which is the form that passes through the PTFE membrane in the permeation cell (PC) and is carried out by the BCP solution (F_3) to the spectrophotometer (SP). The formed

acetic acid causes a color change from purple ($\lambda_{\text{max}} = 590 \text{ nm}$) to yellow ($\lambda_{\text{max}} = 400 \text{ nm}$). The signal, which is monitored at 590 nm, is registered on the chart recorder (R). The organophosphorus pesticide solutions (OPs) and the TMB-4 solution in a phosphate buffer are also introduced by the same valve (V) into flow F_1 . The sample loop consisting of $100 \mu\text{L}$ was composed of a polyethylene tube (1.0 mm i.d.). All of the waste was collected in (W). A Lauda Model RCS RC6 thermostated bath was used to study the influence of the temperature on the enzymatic column.

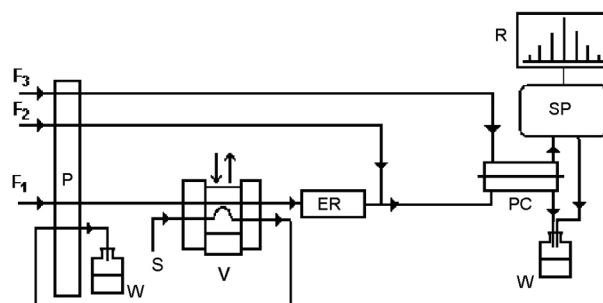


Figure 1. Scheme for the flow injection system used: F_1 = phosphate buffer 0.1 mol L^{-1} pH 7.0; F_2 = sulfuric acid 0.5 mol L^{-1} ; F_3 = bromocresol purple solution $5.0 \times 10^{-5} \text{ mol L}^{-1}$ pH 7.00. $F_1 = F_2 = F_3 = 1.0 \text{ mL min}^{-1}$; P = peristaltic pump; ER = enzymatic reactor; V = sampling valve; PC = permeation cell; S = sample introduction where S is as desired: acetylcholine chloride = $5.0 \times 10^{-3} \text{ mol L}^{-1}$, TMB-4 = 5.0×10^{-3} , standard or sample solutions containing organophosphorus pesticides; W = waste; SP = spectrophotometer, $\lambda = 590 \text{ nm}$; R = recorder.

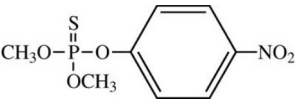
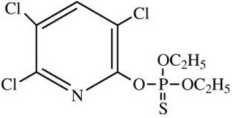
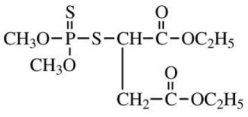
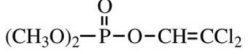
Results and Discussion

The pesticides studied in this work are methyl parathion (*O,O*-dimethyl-*O*-4-nitrophenyl phosphorothioate), chlorpyrifos (*O,O*-diethyl *O*-3,5,6-trichloropyridin-2-yl phosphorothio-ate), malathion (2-(dimethoxyphosphinothioylthio) butanedioic acid diethyl ester) and dichlorvos (2,2-dichlorovinyl dimethyl phosphate). Their molecular formulas are shown in Table 1.

The analytical signal (Figure 2) is calculated from the difference between the height of the signal corresponding to the acetic acid formed from the acetylcholine before the inhibition (peaks 1) of the enzyme and the signal from the formed acetic acid after the inhibition (peaks 3) of the enzyme by the organophosphorus pesticide for the same acetylcholine concentration. The difference between the peak heights corresponding to the uninhibited acetylcholinesterase and those related to the inhibited one is the analytical signal E_1 . Peaks 2 and 4 represent the introduction of the pesticide and the regenerator TMB-4, respectively. Their negative values are due to the presence of CO_2 in the BMP indicator that permeated through the membrane in the direction of the introduced solution, which

causes an increase in the pH and an increase in the intensity of the purple color.

Table 1. The studied pesticides and their molecular formulas²⁸

Pesticide	Molecular formula
Methyl parathion	
Chlorpyrifos	
Malathion	
Dichlorvos	

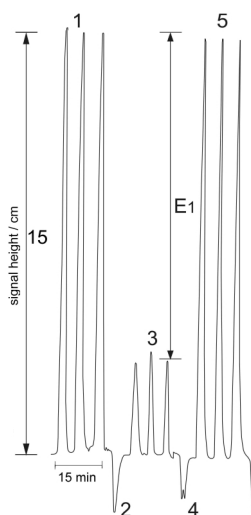


Figure 2. Example of the analytical signal. The group of peaks labeled 1 is related to signal 1 of the acetic acid produced from 5.0×10^{-5} mol L⁻¹ acetylcholine prior to the inhibition of the enzyme by the pesticide. Signal 2 corresponds to the introduction of the pesticide solution in the FIA system. Group 3 is related to the signal after the inhibition of the enzyme by the pesticide. Peak 4 is related to the introduction of the TMB-4 solution into the FIA system. Group 5 is related to the signals for the acetylcholine after the regeneration of the enzyme.

A study of the influences of parameters, such as temperature, acetylcholine chloride concentration, flow rate, sample volume, pH of the reaction medium (phosphate buffer) and sulfuric acid concentration, on the flow injection analysis (FIA) system was performed to optimize the analytical signal. By selecting the best compromise between the signal intensity and the operational characteristics (i.e., cleaning time, temperature control of the reaction, analytical frequency, volume of the

solutions, and concentration of the reagents) of the flow system, the conditions reported in Table 2 were established for the method.

Table 2. Optimized parameters of the FIA system

Parameters of the FIA system	
Wavelength / nm	590
Temperature / °C	25.0
Acetylcholine / (mol L ⁻¹)	5×10^{-3}
Injection loop / μ L	150
Flow rate / (mL min ⁻¹)	1.0
H ₂ SO ₄ / (mol L ⁻¹)	0.5
Sørensen buffer / pH (carrier)	7.5
TMB-4 / (mol L ⁻¹)	5×10^{-5}
Sampling rate / h	12

The determination of the pesticide concentrations was made with and without oxidation using bromine water. The oxidation of the samples was performed by simply adding 1 mL of 20 mmol L⁻¹ bromine water in a 5-mL volumetric flask and filling to the mark with the pesticide solution, without any other pretreatment. The introduction into the analytical flow system was performed after 15 min.

The use of TMB-4 to reactivate the enzyme always exhibited excellent results, which allowed for normal operation of the analytical procedure.

The experiments were performed with 20 mmol L⁻¹ hypochlorite and iodine (I₃⁻) solutions as substitutes for bromine water. However, in contrast to bromine, these reagents irreversibly and cumulatively damage the enzyme during pesticides injection into the flow system, and therefore, the complete recovery of the enzymatic activity does not occur with the TMB-4 regenerator.

The possible direct effect of the bromine water on the enzyme was studied, and no influence of this reagent on the enzyme activity was observed.

Table 3 shows the relationship between the percentage inhibition of the enzyme and the pesticide concentration. The limits of detection were drastically lowered when some drops of bromine water were directly added to the pesticide solution. The resulting compounds are much more vigorous inhibitors of acetylcholinesterase, and therefore, the analytical signal significantly increased. Morita and Kumaran²⁹ reported that organophosphorus pesticide oxidation with bromine water changes the P=S group to the corresponding P=O group, and these compounds typically exhibit increased enzyme inhibition. However, this explanation does not clarify the results for dichlorvos, which contains a P=O group in the original molecule.

Duirk *et al.*³⁰ have elucidated the distribution of chlorpyrifos in the presence of aqueous hypochlorite solution in the pH range of 6.3 to 11. They observed the fast oxidation of chlorpyrifos (CP) by OCI^- , which resulted in a more toxic compound (i.e., chlorpyrifos-oxon (CPO)). Duirk *et al.*³⁰ also studied the hydrolysis of dimethylformamide (DMF) for CP and 3,5,6-trichloro-2-pyridinol (TCP). In a study of the degradation products of organophosphorus pesticides, Kralj *et al.*³¹ observed that, for the pesticide chlorpyrifos, a product analogous to chlorpyrifos-oxon was detected. Duirk *et al.*³⁰ also reported that one of the degradation products is chlorpyrifos-oxon.

One possible suggestion for dichlorvos is that bromine addition occurs at the carbon-carbon double bond. Certainly, a more detailed study must be performed to explain the increase in the toxicity of these types of pesticides after oxidation with bromine water, but this work is beyond the scope of the current work.

Table 3 shows the relative percent inhibition of the aqueous solutions for the four pesticides on the acetylcholinesterase. The solutions in fresh orange juice were also studied. The inhibition was studied in the presence and absence of bromine water in all of the cases. The percent inhibition was calculated for a pesticide concentration according to $\%(\text{inhibition}) = ((E_1/E_2) \times 100)$, where E_1 is the difference between the two signals before and after inhibition and E_0 is the signal before inhibition.

The higher toxicity of the pesticides oxidized by the addition of bromine is evident, which is reflected in the increase in the inhibition of the enzyme. In the more dramatic case of dichlorvos, concentrations with a magnitude of $10^{-10} \text{ mol L}^{-1}$ can be detected.

To test the method in a real matrix, orange juice was separately spiked with known quantities of methyl parathion, chlorpyrifos, malathion and dichlorvos. The results are shown in Table 3.

Table 3. Inhibition of the enzyme acetylcholinesterase as functions of the concentration of the pesticides methyl parathion, chlorpyrifos, malathion and dichlorvos. Comparison of the inhibition power of the pesticides with respect to dichlorvos arbitrarily taken as reference. Determination of these pesticides in fresh orange juice purposely contaminated

Conc. / (mol L ⁻¹)	Pesticide methyl malathion										Pesticide chlorpyrifos									
	Not oxidized					Oxidized with bromine water					Not oxidized					Oxidized with bromine water				
	p ^a	± sd ^b	p	± sd	dp ^c	± sd	oj ^d	± sd	ojdp ^e	± sd	p	± sd	p	± sd	dp	± sd	oj	± sd	ojdp	± sd
1.0×10^{-10}	0.0	—	0.0	—	0.00	—	—	—	—	—	0.0	—	0.0	—	0.00	—	—	—	—	—
1.0×10^{-9}	0.0	—	2.3	0.7	0.03	0.01	—	—	—	—	0.0	—	0.0	—	0.00	—	—	—	—	—
1.0×10^{-8}	0.0	—	15.0	2.2	0.17	0.02	13.5	1.1	0.16	0.01	0.0	—	10.2	0.8	0.12	0.01	8.3	1.0	0.10	0.01
1.0×10^{-7}	0.0	—	26.2	1.4	0.31	0.02	—	—	—	—	0.0	—	27.7	1.7	0.32	0.02	—	—	—	—
1.0×10^{-6}	0.0	—	50.0	1.0	0.58	0.02	47.4	1.1	0.55	0.02	0.0	—	45.4	1.3	0.53	0.02	42.5	1.4	0.49	0.02
1.0×10^{-5}	7.8	1.0	71.0	0.8	0.83	0.02	—	—	—	—	0.0	—	64.8	1.3	0.75	0.02	—	—	—	—
1.0×10^{-4}	19.0	1.0	89.1	1.6	1.04	0.03	—	—	—	—	8.3	0.5	83.6	0.8	0.97	0.02	—	—	—	—
1.0×10^{-3}	31.6	1.5	97.3	0.8	1.13	0.02	—	—	—	—	20.3	0.8	95.0	0.8	1.11	0.02	—	—	—	—
Conc. / (mol L ⁻¹)	Pesticide malathion										Pesticide dichlorvos									
	Not oxidized					Oxidized with bromine water					Not oxidized					Oxidized with bromine water				
	p	± sd	p	± sd	dp	± sd	oj	± sd	ojdp ^e	± sd	p	± sd	p	± sd	dp	± sd	oj	± sd	ojdp	± sd
1.0×10^{-10}	0.0	—	0.0	—	0.00	—	—	—	—	—	0.0	—	6.1	1.1	0.07	0.01	—	—	—	—
1.0×10^{-9}	0.0	—	0.0	—	0.00	—	—	—	—	—	0.0	—	8.8	1.3	0.10	0.01	7.2	1.1	0.08	0.01
1.0×10^{-8}	0.0	—	0.0	—	0.00	—	—	—	—	—	0.0	—	17.9	0.8	0.21	0.01	—	—	—	—
1.0×10^{-7}	0.0	—	3.2	1.0	0.04	0.01	—	—	—	—	0.0	—	25.6	1.2	0.30	0.01	—	—	—	—
1.0×10^{-6}	0.0	—	8.4	2.0	0.10	0.02	7.2	1.5	0.08	0.01	0.0	—	38.0	1.3	0.44	0.02	52.1	1.3	0.61	0.02
1.0×10^{-5}	0.0	—	16.3	1.6	0.19	0.02	—	—	—	—	4.2	0.8	53.5	1.3	0.62	0.02	—	—	—	—
1.0×10^{-4}	0.0	—	52.8	2.5	0.61	0.03	49.9	2.0	0.58	0.02	14.8	0.8	73.2	2.4	0.85	0.03	—	—	—	—
1.0×10^{-3}	4.3	0.6	75.0	2.2	0.87	0.03	—	—	—	—	24.6	0.8	86.0	0.8	1.00	0.01	—	—	—	—

^ap = percentage of inhibition; ^bsd = standard deviation; ^cdp: inhibition of the pesticide proportional to dichlorvos $1.0 \times 10^{-3} \text{ mol L}^{-1}$ inhibition; ^doj: pesticide spiked in fresh orange juice; ^eojdp: inhibition of the pesticide dissolved in orange juice proportional to dichlorvos $1.0 \times 10^{-3} \text{ mol L}^{-1}$ inhibition.

No similar equations correlating the inhibition of the oxidized forms of the pesticides and concentration were observed for the four organophosphorus compounds. Therefore, for comparison, dichlorvos was used as the reference because it exhibits inhibitory action over a wider range of concentrations. For this comparison, the parameter dichlorvos proportion (dp), which is the rate between the inhibition of the enzyme by a pesticide in a given concentration and the inhibition caused by 1.0×10^{-3} mol L⁻¹ dichlorvos, is introduced. The calculated values are also shown in Table 3.

This method provides a good indication of the level of toxicity of a sample containing an organophosphorus pesticide even without specifically knowing which compound is present, allowing for rapid medical decisions due to intoxication. In this type of analysis, a standard sample of 1.0×10^{-3} mol L⁻¹ dichlorvos must be analyzed for comparison.

Conclusion

Based on the observed results, the proposed method is reliable, low cost, rapid and easily performed. This method also exhibits very good detection because very low concentrations of organophosphorus pesticides, for which acetylcholinesterase is a specific sensor, were detected. Therefore, the method can be applied for the detection of these types of substances.

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